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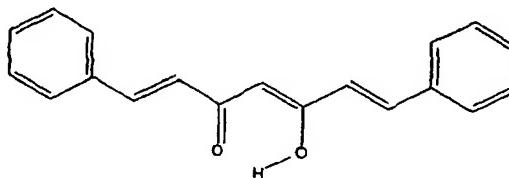
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**WO 03/035007 A2**

(54) Title: CURCUMINOID COMPOSITIONS EXHIBITING SYNERGISTIC INHIBITION OF THE EXPRESSION AND/OR ACTIVITY OF CYCLOOXYGENASE-2



(57) Abstract: A novel formulation is provided that serves to inhibit the inflammatory response in animals. The formulation comprises, as a first component an effective amount of a curcuminoid species and an effective amount of a second component selected from the group consisting of an alpha-acid species or a beta-acid species or derivatives thereof. The composition provides synergistic anti-inflammatory effects in response to physical or chemical injury or abnormal immune stimulation due to a biological agent or unknown etiology.

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PATENT

**CURCUMINOID COMPOSITIONS EXHIBITING SYNERGISTIC INHIBITION OF THE EXPRESSION  
AND/OR ACTIVITY OF CYCLOOXYGENASE-2**

Background of the Invention

5    Field of the Invention

The present invention relates generally to a composition exhibiting synergistic inhibition of the expression and/or activity of inducible cyclooxygenase-2 (COX-2). The composition can function synergistically to inhibit the inducibility and/or activity of inducible cyclooxygenase (COX-2) with little or no significant effect on constitutive cyclooxygenase (COX-1).

10

Description of the Related Art

Inflammatory diseases affect more than fifty million Americans. As a result of basic research in molecular and cellular immunology over the last ten to fifteen years, approaches to diagnosing, treating and preventing these immunologically-based diseases has been dramatically altered. One example of this is the 15 discovery of an inducible form of the cyclooxygenase enzyme. Constitutive cyclooxygenase (COX), first purified in 1976 and cloned in 1988, functions in the synthesis of prostaglandins (PGs) from arachidonic acid (AA). Three years after its purification, an inducible enzyme with COX activity was identified and given the name COX-2, while constitutive COX was termed COX-1.

COX-2 gene expression is under the control of pro-inflammatory cytokines and growth factors. Thus, 20 the inference is that COX-2 functions in both inflammation and control of cell growth. While COX-2 is inducible in many tissues, it is present constitutively in the brain and spinal cord, where it may function in nerve transmission for pain and fever. The two isoforms of COX are nearly identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations. Protective PGs, which preserve the integrity of the stomach lining and maintain normal renal function in a compromised 25 kidney, are synthesized by COX-1. On the other hand, PGs synthesized by COX-2 in immune cells are central to the inflammatory process.

An ideal formulation for the treatment of inflammation would inhibit the induction and activity of COX-2 without affecting the activity of COX-1. However, conventional non-steroidal and steroidal anti-inflammatory drugs lack the specificity of inhibiting COX-2 without affecting COX-1 and are at risk to cause damages on the 30 gastrointestinal system when used for extended periods.

Numerous studies have shown that the relative incidence of gastrointestinal (GI) side effects can be correlated to the relative COX-2 specificity of the agents. The higher the specificity for COX-2 over COX-1, the lower the incidence of GI upset. Thus, aspirin, with a COX-2 specificity of only 0.6, produces a greater incidence of GI distress than curcuminoids, with a reported COX-2 specificity of nearly 3.0. However, the 35 generally accepted COX-2 specificity necessary to significantly reduce the probability of GI upset is 5.0.

Therefore, it would be useful to identify a composition that would specifically inhibit or prevent the expression of COX-2 enzymatic activity, while having little or no effect on COX-1 metabolism so that these could be used at sufficiently low doses or at current clinical doses, with no adverse side effects.

Medical doctors generally utilize non-steroidal and steroidal anti-inflammatory drugs for treatment of 5 osteoarthritis. These drugs, however, are not well adapted for long-term therapy because they not only lack the ability to promote and protect cartilage; they can actually lead to degeneration of cartilage or reduction of its synthesis. Moreover, most non-steroidal, anti-inflammatory drugs damage the gastrointestinal system when used for extended periods. Thus, new treatments for arthritis are urgently needed.

The joint-protective properties of glucosamine would make it an attractive therapeutic agent for 10 osteoarthritis except for two drawbacks: (i) the rate of response to glucosamine treatment is slower than for treatment with anti-inflammatory drugs, and (ii) glucosamine may fail to fulfill the expectation of degenerative remission. In studies comparing glucosamine with non-steroidal anti inflammatory agents, for example, a double-blinded study comparing 1500 mg glucosamine sulfate per day with 1200 mg ibuprofen, demonstrated 15 that pain scores decreased faster during the first two weeks in the ibuprofen patients than in the glucosamine-treated patients. However, the reduction in pain scores continued throughout the trial period in patients receiving glucosamine and the difference between the two groups turned significantly in favor of glucosamine by week eight. Lopes Vaz, A., *Double-blind clinical evaluation of the relative efficacy of ibuprofen and glucosamine sulphate in the management of osteoarthritis of the knee in outpatients*, 8 Curr. Med Res Opin. 145-149 (1982). Thus, glucosamine may relieve the pain and inflammation of arthritis, but at a slower rate 20 than the available anti-inflammatory drugs.

Moreover, the currently available glucosamine formulations have not been formulated to optimally attack and alleviate the underlying causes of osteoarthritis and rheumatoid arthritis. Also, as with many commercially-available herbal and dietary supplements, the available formulations do not have a history of usage, nor controlled clinical testing, which might ensure their safety and efficacy.

An ideal formulation for the normalization of cartilage metabolism or treatment of osteoarthritis would 25 provide adequate chondroprotection with potent antiinflammatory activity. The optimal dietary supplement for osteoarthritis should enhance the general joint rebuilding qualities offered by glucosamine and attenuate the inflammatory response without introducing any harmful side effects. It should be inexpensively manufactured and comply with all governmental regulations.

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#### Summary of the Invention

Thus, it would be useful to identify a natural formulation of compounds that would specifically inhibit or prevent the synthesis of prostaglandins by COX-2 with little or no effect on COX-1. Such a formulation, which would be useful for preserving the health of joint tissues, for treating arthritis or other inflammatory 35 conditions, has not previously been discovered. The term "specific or selective COX-2 inhibitor" embraces

compounds or mixtures of compounds that selectively inhibit COX-2 over COX-1. Preferably, the compounds have a median effective concentration for COX-2 inhibition that is minimally five times greater than the median effective concentration for the inhibition of COX-1. For example, if the median inhibitory concentration for COX-2 of a test formulation was 0.2 µg/mL, the formulation would not be considered COX-2 specific unless 5 the median inhibitory concentration for COX-1 was equal to or greater than 1 µg/mL.

The preferred embodiments provide a composition comprising, as a first component, a curcuminoid species and a second compound that can synergistically enhance the anti-inflammatory effect of the curcuminoid. The composition comprises an effective amount of a first component comprising a curcuminoid species and a second component comprising a member selected from the group consisting of an alpha acid, 10 a beta acid, and derivatives thereof. In a certain embodiment, the alpha acid is humulone. In another embodiment, the beta acid is lupulone. In another embodiment, the curcuminoid is curcumin.

In a certain embodiment, the composition further comprises a member selected from the group consisting of glucosamine and chondroitin sulfate.

The preferred embodiments also provide a method of treating inflammation or inflammation-based 15 diseases in an animal which comprises providing to the animal suffering symptoms of inflammation a composition comprising an effective amount of a first component comprising a curcuminoid species and a second component comprising a member selected from the group consisting of an alpha acid, a beta acid, and derivatives thereof.

The preferred embodiments also provide a method of reducing the symptoms of osteoarthritis in an 20 animal which comprises providing to the animal suffering symptoms of inflammation a composition comprising an effective amount of a first component comprising a curcuminoid species and a second component comprising a member selected from the group consisting of an alpha acid, a beta acid, and derivatives thereof.

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#### Brief Description of the Drawings

Figures 1[A]-[F] illustrate the general chemical structure of [A] the curcuminoid genus and [B], [C], [D], [E] and [F], respectively, as curcumin, demethoxycurcumin, bisdemethoxycurcumin, the cis-trans geometrical isomer of curcumin, and cyclocurcumin as species within that genus.

Figures 2[A] and [B], respectively, illustrate the general chemical structures of the alpha-acid genus 30 and humulone as a species within that genus.

Figures 3[A] and [B], respectively, illustrate the general chemical structures of the beta-acid genus and lupulone

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Detailed Description of the Preferred Embodiment

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

5       The preferred embodiments provide compositions having a synergistic inhibitory effect on the expression and/or activity of COX-2. More particularly, the composition comprises, as a first component, a curcuminoid and, as a second component, a member selected from the group consisting of an alpha-acid, a beta-acid, and derivatives thereof, as more specifically described below. The composition provided by the preferred embodiments can be formulated as a dietary supplement or therapeutic composition. The 10 composition can function synergistically to inhibit the inducibility and/or activity of COX-2 with no significant effect on COX-1.

As used herein, the term "dietary supplement" refers to compositions consumed to affect structural or functional changes in physiology. The term "therapeutic composition" refers to any compounds administered to treat or prevent a disease.

15      As used herein, the terms "curcuminoid" and "active curcuminoid" refer to species within the curcuminoid genera that is capable of inhibiting the inducibility and/or activity of COX-2 while having little or no effect on COX-1 or is capable of inhibiting or reducing the severity of an inflammatory response. The curcuminoid can be extracted from natural products or chemically synthesized.

20      A yellow pigmented fraction isolated from the rhizomes of *Curcuma longa* contains curcuminoids belonging to the dicinnamoyl methane group. Curcuminoids are present to the extent of 3 to 5 percent. They are considered the most important active ingredients and are believed to be responsible for the biological activity of *Curcuma longa*. Though their major activity is anti-inflammatory, curcuminoids have been reported to possess antioxidant, anti-allergic, wound healing, antispasmodic, antibacterial, antifungal and antitumor activity as well. Curcumin (Fig. 1B) was isolated in 1815 and structurally defined in 1910. Other curcuminoids 25 isolated from *Curcuma longa* include demethoxycurcumin (Fig. 1C), bisdemethoxycurcumin (Fig. 1D), a cis-trans geometrical isomer of curcumin (Fig. 1E), and cyclocurcumin (Fig 1F). Curcuminoids may be found in other botanicals in addition to *Curcuma longa*, such as *Curcuma xanthorrhiza* and *Curcuma zedoaria*.

25      Curcuminoids are well known for their anti-inflammatory activity. Tumeric is one of the oldest anti-inflammatory drugs used in Ayurvedic medicine. The anti-inflammatory activity of curcuminoids has been evaluated in inflammatory reaction models such as chemical or physical irritants like carrageenin, cotton pellets, formaldehyde and the granuloma pouch. Human, double-blinded, clinical trials have demonstrated efficacy in rheumatoid arthritis at a dose of 1200 mg curcuminoids/day for five to six weeks. At these doses, however, signs of gastrointestinal (GI) discomfort and stomach irritation are frequently reported. The GI upset and stomach irritation caused by high doses of curcuminoids may be due to the fact that curcuminoids act on 30 prostaglandin production in a manner similar to that of aspirin and aspirin-like anti-inflammatory agents.

Preferably, the curcuminoid genus, as represented by FIG.1[A] and specifically exemplified by curcumin in FIG.1[B] is a pharmaceutical grade botanical extract such as can be obtained commercially, for example, from Sabinsa (121 Ethel Road West, Piscataway, NJ). Other curcuminoids that may be employed include demethoxycurcumin (FIG 1[C]), bisdemethoxycurcumin (FIG. 1[D]), a cis-trans curcumin (Fig 1E), and 5 cyclocurcumin (FIG 1F). The curcuminoid used can be readily obtained from *Curcuma longa* L. Pharmaceutical grade curcuminoid extract is standardized to have a curcuminoid content of greater than about 70 percent. The pharmaceutical, botanical grade extract preferably should have passed extensive safety and efficacy procedures. As employed in the preferred embodiments, the extract has a curcuminoid content of about 1 to 99 percent by weight. Preferably, the minimum curcuminoid content is about 70 percent 10 by weight. Alternatively, the curcuminoid may be synthesized using standard techniques known in chemical synthesis.

As used herein, the term "hop extract" refers to the solid material resulting from (1) exposing a hops plant product to a solvent, (2) separating the solvent from the hops plant product, and (3) eliminating the solvent.

15 As used herein, the term "solvent" refers to a liquid of aqueous or organic nature possessing the necessary characteristics to extract solid material from the hop plant product. Examples of solvents would include, but are not limited to, water, steam, superheated water, methanol, ethanol, hexane, chloroform, liquid CO<sub>2</sub>, liquid N<sub>2</sub> or any combinations of such materials.

20 As used herein, the term "CO<sub>2</sub> extract" refers to the solid material resulting from exposing a hops plant product to a liquid or supercritical CO<sub>2</sub> preparation followed by the removal of the CO<sub>2</sub>.

As used herein, the term "alpha-acids" refers to compounds isolated from hops plant products including, but not limited to, humulone, cohumulone, isohumulone, isoprehumulone, hulupone, adhumulone, xanthohumol A and xanthohumol B.

25 As used herein, the term "beta-acids" refers to compounds collectively known as lupulones including, but not limited to, lupulone, colupulone, adlupulone, tetrahydroisohumulone, and hexahydrocolupulone.

As used herein, the term "essential oil fraction" refers to a complex mixture of components comprising myrcene, humulene, beta-caryophyleen, undecane-2-on, and 2-methyl-but-3-en-ol.

As used herein, the term "fats" refers to triacylglycerol esters of fatty acids.

As used herein, the term "waxes" refers to triacylglycerol ethers or esters of extremely long chain 30 (>25 carbons) fatty alcohols or acids.

Hop extraction in one form or another goes back over 150 years to the early nineteenth century when extraction in water and ethanol was first attempted. Even today an ethanol extract is available in Europe, but by far the predominant extracts are organic solvent extracts (hexane) and CO<sub>2</sub> extracts (supercritical and liquid). CO<sub>2</sub> (typically at 60 bars pressure and 5 to 10°C) is in a liquid state and is a relatively mild, non-polar 35 solvent highly specific for hop soft resins and oils. Beyond the critical point, typically at 300 bars pressure and

60°C, CO<sub>2</sub> has the properties of both a gas and a liquid and is a much stronger solvent. The approximate components of the various extracts is compared in Table 1.

TABLE 1. Hop Extracts (Percent W/W)

Component	Hops	Organic Solvent Extract	Super-Critical CO <sub>2</sub>	Liquid CO <sub>2</sub>
Total resins	12 - 20	15 - 60	75 - 90	70 - 95
Alpha-acids	2 - 12	8 - 45	27 - 55	30 - 60
Beta-acids	2 - 10	8 - 20	23 - 33	15 - 45
Essential oils	0.5 - 1.5	0 - 5	1 - 5	2 - 10
Hard resins	2 - 4	2 - 10	5 - 11	None
Tannins	4 - 10	0.5 - 5	0.1 - 5	None
Waxes	1 - 5	1 - 20	4 - 13	0 - 10
Water	8 - 12	1 - 15	1 - 7	1 - 5

5

At its simplest, hop extraction involves milling, pelleting and re-milling the hops to spread the lupulin, passing a solvent through a packed column to collect the resin components and finally, removal of the solvent to yield a whole or "pure" resin extract.

10 The main organic extractants are strong solvents and in addition to virtually all the lupulin components, they extract plant pigments, cuticular waxes, water and water-soluble materials.

Supercritical CO<sub>2</sub> is more selective than the organic solvents and extracts less of the tannins and waxes and less water and hence water-soluble components. It does extract some of the plant pigments like chlorophyll but less than the organic solvents do. Liquid CO<sub>2</sub> is the most selective solvent used commercially for hops and hence produces the most pure whole resin and oil extract. It extracts none of the hard resins or 15 tannins, much lower levels of plant waxes, no plant pigments and less water and water-soluble materials.

As a consequence of this selectivity and the milder solvent properties, the absolute yield of liquid CO<sub>2</sub> extract per unit weight of hops is less than when using the other mentioned solvents. Additionally, the yield of alpha acids with liquid CO<sub>2</sub> (about 89-93%) is lower than that of supercritical CO<sub>2</sub> (about 91-94%) or the organic solvents (about 93-96%). Following extraction there is the process of solvent removal, which for 20 organic solvents involves heating to cause volatilization. Despite this, trace amounts of solvent do remain in the extract. The removal of CO<sub>2</sub>, however, simply involves a release of pressure to volatilize the CO<sub>2</sub>.

In the preferred embodiments, the alpha acid, beta acid, or derivative thereof can be extracted from hops or chemically synthesized. Preferably, the alpha acid, beta acid, or derivative thereof is extracted from hops, more preferably extracted by supercritical CO<sub>2</sub>.

25 Preferably, the alpha-acid genus, as represented by FIG. 2 [A] and specifically exemplified by humulone in FIG. 2 [B], and the beta-acid genus, as represented by FIG. 3 [A] and specifically exemplified by

Iupulone (FIG. 3 [B]) is a pharmaceutical grade preparation such as can be obtained commercially, for example, from Hopunion. (Yakima, WA).

- The identification of humulone from hops extract as an inhibitor of bone resorption is reported in Tobe, H. et al. 1997. [Bone resorption Inhibitors from hop extract. Biosci. Biotech. Biochem 61(1)158-159.]
- 5 Later studies by the same group characterized the mechanism of action of humulone as inhibition of COX-2 gene transcription following TNFalpha stimulation of MC3T3 -E1 cells [Yamamoto, K. 2000. Suppression of cyclooxygenase-2 gene transcription by humulon of bee hop extract studied with reference to glucocorticoid. FEBS Letters 465:103-106]. However, these references disclose the use of humulone alone for the applications of osteoporosis and COX-2 gene transcription.
- 10 The preferred embodiments provide for modifying the curcuminoid molecule to achieve greater efficacy and lower toxicity and adding a second component that acts in a synergistic manner. Therefore, preferred embodiments relate to a discovery that when combining a curcuminoid with a second molecule selected from the group consisting of a alpha-acid, a beta-acid, and derivatives thereof, the combination produces a synergistic effect in a target cell. One such synergistic response would be the specific inhibition of
- 15 inducible COX-2.

Representative species within each genus are listed in Table 2. Of the species listed under each genus in Table 2, those containing at least one asterisk (\*) are preferred and those containing two asterisks (\*\*) are particularly preferred.

20

TABLE 2. Components of Composition

CURCUMINOIDS	ALPHA-ACIDS	BETA ACIDS
Curcumin**	Humulone**	Lupulone**
Demethoxycurcumin**	Cohumulone*	Colupulone*
Bisdemethoxycurcumin**	Isohumulone*	Adlupulone*
Cis-trans curcumin*	Isoprehumulone*	Tetrahydroisohumulone*
Cyclocurcumin*	Hulupone*	Hexahydrocolupulone*
	Adhumulone*	Dihydro-isohumulone*
	Xanthohumulone A*	
	Xanthohumulone B*	

Preferably, the preferred embodiments utilize active curcuminoid and active ingredients of hop extract or derivatives thereof. As used herein, the term "active curcuminoid", "active ingredient of hop extract" or  
25 derivatives thereof refers to naturally occurring or synthetic derivatives of species within the scope of the

respective genera that are capable of inhibiting the inducibility and/or activity of COX-2 while having little or no effect on COX-1 or are capable of inhibiting or reducing the severity of an inflammatory response.

The preferred embodiments can also use conjugates of curcuminoids, alpha- and beta-acids or derivatives thereof. "Conjugates" of curcuminoids, alpha- and beta-acids or derivatives thereof means curcuminoids, alpha-acids, and beta-acids covalently bound or conjugated to a member selected from the group consisting of mono- or di- saccharides, amino acids, sulfates, succinate, acetate and glutathione. Preferably, the mono- or di- saccharide is a member selected from the group consisting of glucose, mannose, ribose, galactose, rhamnose, arabinose, maltose, and fructose.

A certain embodiment is a composition comprising an effective amount of curcumin and at least one compound selected from the group consisting of humulone and lupulone.

The inhibition of the activity of the COX-2 enzyme by alpha-acids or beta-acids can provide a dual, synergistic effect with curcuminoids. Thus, the second compound selected from the group consisting of alpha-acids and beta-acids can increase the anti-inflammatory activity of the curcuminoids. The result of the compositions of the preferred embodiments is a more selective effect on the activity of COX-2 at lower doses of curcuminoids than would normally be required. By decreasing the dose of curcuminoids to achieve the desired COX-2 inhibition, the probability of side effects from this compound decreases almost exponentially. The second compound selected from the group consisting of alpha-acids and beta-acids can provide benefits, such as, but not limited to, hepatoprotection, antitumor promotion, antihyperlipidemia, antihyperglycemia and protection against ulcer formation from COX-1 inhibition by the curcuminoids.

Preferably, a daily dose (mg/kg-day) of the preferred dietary supplement would be formulated to deliver, per kg body weight of the animal, about 0.001 to about 30.0 mg curcuminoids, and about 0.5 to about 20.0 mg alpha-acids or beta-acids.

The composition of the preferred embodiments for topical application would contain one of the following: about 0.001 to about 1 wt%, preferably about 0.01 to about 1 wt% curcuminoids, and about 0.025 to about 1 wt%, preferably about 0.05 to about 1wt% alpha-acids or beta-acids.

The composition of the preferred embodiments would produce serum concentrations in the following range: about 0.0001 to about 10  $\mu$ M of curcuminoids, and about 0.001 to about 10  $\mu$ M alpha-acids or beta-acids.

In the preferred embodiments, the composition can further comprise glucosamine or chondroitin sulfate. Glucosamine is generally accepted as being effective and safe for treating osteoarthritis. Therefore, the compositions that further comprise glucosamine or chondroitin sulfate can aid in normalizing joint function or reducing the symptoms of osteoarthritis.

In addition to the combination of curcuminoids and alpha-acids, beta-acids or derivatives, the present composition for dietary application can include various additives such as, but not limited to, other natural components of intermediary metabolism, antioxidants, vitamins, minerals, proteins, fats, carbohydrates, and

aminosugars, as well as inert ingredients such as, but not limited to, talc and magnesium stearate, that are standard excipients in the manufacture of tablets and capsules.

The composition of the preferred embodiments can further comprise a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, sweeteners and the like. These pharmaceutically acceptable carriers can be prepared from a wide range of materials including, but not limited to, diluents, binders and adhesives, lubricants, disintegrants, coloring agents, bulking agents, flavoring agents, sweetening agents and miscellaneous materials such as buffers and absorbents that may be needed in order to prepare a particular therapeutic composition. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in the preferred embodiments is contemplated. In one embodiment, talc and magnesium stearate are included in the formulation. Preferable components are Astac Brand 400 USP talc powder and the veritable grade of magnesium stearate. Other ingredients known to affect the manufacture of this composition as a dietary bar or functional food can include flavorings, sugars, amino-sugars, proteins and/or modified starches, as well as fats and oils.

The dietary supplements, lotions or therapeutic compositions of the preferred embodiments can be formulated in any manner known by one of skill in the art. In one embodiment, the composition is formulated into a capsule or tablet using techniques available to one of skill in the art. In capsule or tablet form, the recommended daily dose for an adult human or animal would preferably be contained in one to six capsules or tablets. However, the present compositions may also be formulated in other convenient forms, such as an injectable solution or suspension, a spray solution or suspension, a lotion, gum, lozenge, food or snack item. Food, snack, gum or lozenge items can include any ingestible ingredient, including sweeteners, flavorings, oils, starches, proteins, fruits or fruit extracts, vegetables or vegetable extracts, grains, animal fats or proteins. Thus, the present compositions can be formulated into cereals, snack items such as chips, bars, gumdrops, 25 chewable candies or slowly dissolving lozenges. The preferred embodiments contemplate treatment of all types of inflammation-based diseases, both acute and chronic. The present formulation reduces the inflammatory response and thereby promotes healing of, or prevents further damage to, the affected tissue. A pharmaceutically acceptable carrier may also be used in the present compositions and formulations.

According to the preferred embodiments, the animal may be a member selected from the group consisting of humans, non-human primates, dogs, cats, birds, horses, ruminants or other warm blooded animals. The preferred embodiments are directed primarily to the treatment of human beings. Administration can be by any method available to the skilled artisan, for example, by oral, topical, transdermal, transmucosal, or parenteral routes.

TABLE 3 below provides a list of diseases in which COX-2 enzyme expression and activity may play a role and therefore are appropriate targets for normalization or treatment by the compositions of the preferred embodiments.

5

TABLE 3. COX-2 Associated Diseases

DISEASE	TISSUE
Addison's Disease	Adrenal
Allergies	Inflammatory cells
Alzheimer Disease	Nerve cells
Arthritis	Inflammatory cells
Atherosclerosis	Vessel wall
Colon Cancer	Intestine
Crohn's Disease	Intestine
Diabetes (type I)/type II	Pancreas
Eczema	Skin/Inflammatory cells
Graves' Disease	Thyroid
Guillain-Barre Syndrome	Nerve cells
Inflammatory Bowel Disease	Intestine
Leukemia	Immune cells
Lymphomas	Immune cells
Multiple Sclerosis	Nerve cells
Myasthenia Gravis	Neuromuscular junction
Osteoarthritis	Joint lining
Psoriasis	Skin
Primary Biliary Cirrhosis	Liver
Rheumatoid Arthritis	Joint lining
Solid Tumors	Various
Systemic Lupus Erythematosis	Multiple tissues
Uveitis	Eye

The discovery of COX-2 has made possible the design of drugs that reduce inflammation without removing the protective PGs in the stomach and kidney made by COX-1. Compositions of the preferred embodiments would be useful for, but not limited to, the treatment of inflammation in a subject, and for  
10 treatment of other inflammation-associated disorders, such as, as an analgesic in the treatment of pain and headaches, or as an antipyretic for the treatment of fever. Compositions of the preferred embodiments would be useful in treating inflammation in such diseases as vascular diseases, migraine headaches, periarthritis nodosa, thyroiditis, aplastic anemia, Hodgkin's disease, sclerodema, rheumatic fever, type I diabetes, myasthenia gravis, multiple sclerosis, sarcoidosis, nephrotic syndrome, Behchet's syndrome, polymyositis,  
15 gingivitis, hypersensitivity, swelling occurring after injury, myocardial ischemia and the like. Compositions of the preferred embodiments are useful as anti-inflammatory agents with the additional benefit of having significantly less harmful side effects.

The preferred embodiments can also provide a composition of matter to increase the rate at which glucosamine or chondroitin sulfate function to normalize joint movement or reduce the symptoms of osteoarthritis. For example, compositions of the preferred embodiments would be useful to treat arthritis, including but not limited to rheumatoid arthritis, spondyloarthropathies, gouty arthritis, osteoarthritis, systemic lupus erythematosus, and juvenile arthritis.

Such compositions of the preferred embodiments would also be useful in the treatment of asthma, bronchitis, menstrual cramps, tendonitis, bursitis, and skin related conditions such as psoriasis, eczema, burns and dermatitis. Compositions of the preferred embodiments also would be useful to treat gastrointestinal conditions such as inflammatory bowel disease, Crohn's disease, gastritis, irritable bowel syndrome and ulcerative colitis and for the prevention or treatment of cancer such as colorectal cancer.

The compositions of the preferred embodiments would also be useful in the treatment of ophthalmic diseases, such as retinopathies, conjunctivitis, uveitis, ocular photophobia, and of acute injury to the eye tissue. The compounds would also be useful in the treatment of pulmonary inflammation, such as that associated with viral infections and cystic fibrosis. The compounds would also be useful for the treatment of certain nervous system disorders such as cortical dementias including Alzheimer's disease. As inhibitors of COX-2 mediated biosynthesis of PGE2, these compositions would also be useful in the treatment of allergic rhinitis, respiratory distress syndrome, endotoxin shock syndrome, atherosclerosis, and central nervous system damage resulting from stroke, ischemia and trauma.

The following examples are intended to illustrate but not in any way limit the preferred embodiments:

20

#### EXAMPLE 1

##### Synergistic Inhibition of Prostaglandin E2 Production in Murine B Cells by Curcuminoids and an Extract of Hops

This example illustrates the superior COX-2 inhibitory potency and selectivity of the combination of curcuminoids and hops extract of the preferred embodiments compared to curcuminoids alone.

##### Inhibition of COX-2 Mediated Production of PGE2 in RAW 264.7 Cells

Equipment - balancer, analytical, Ohaus Explorer (Ohaus Model #EO1140, Switzerland), biosafety cabinet (Forma Model #F1214, Marietta, Ohio), pipettor, 100 to 1000 µL (VWR Catalog #4000-208, Rochester, NY), cell hand tally counter (VWR Catalog #23609-102, Rochester, NY), CO<sub>2</sub> incubator (Forma Model #F3210, Marietta, Ohio), hemacytometer (Hausser Model #1492, Horsham, PA), microscope, inverted (Leica Model #DM IL, Wetzlar, Germany), multichannel pipettor, 12-Channel (VWR Catalog #53501-662, Rochester, NY), Pipet Aid (VWR Catalog #53498-103, Rochester, NY), Pipettor, 0.5 to 10 µL (VWR Catalog #4000-200, Rochester, NY), pipettor, 100 to 1000 µL (VWR Catalog #4000-208, Rochester, NY), pipettor, 2 to 20 µL (VWR Catalog #4000-202, Rochester, NY), pipettor, 20 to 200 µL (VWR Catalog #4000-204,

Rochester, NY), PURELAB Plus Water Polishing System (U.S. Filter, Lowell, MA), refrigerator, 4°C (Forma Model #F3775, Marietta, Ohio), vortex mixer (VWR Catalog #33994-306, Rochester, NY), water bath (Shel Lab Model #1203, Cornelius, OR).

*Cells, Chemicals, Reagents and Buffers* - Cell scrapers (Corning Catalog #3008, Corning, NY),  
5 dimethylsulfoxide (DMSO) (VWR Catalog #5507, Rochester, NY), Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech Catalog #10-013-CV, Herndon, VA), fetal bovine serum, heat inactivated (FBS-HI) (Mediatech Catalog #35-011-CV, Herndon, VA), lipopolysaccharide (LPS)(Sigma Catalog #L-2654, St. Louis, MO), microfuge tubes, 1.7 mL (VWR Catalog #20172-698, Rochester, NY), penicillin/streptomycin (Mediatech Catalog #30-001-CI, Herndon, VA), pipet tips for 0.5 to 10 µL pipettor (VWR Catalog #53509-138, Rochester, 10 NY), pipet tips for 100-1000 µL pipettor (VWR Catalog #53512-294, Rochester, NY), pipet tips for 2-20 µL and 20-200 µL pipettors (VWR Catalog #53512-260, Rochester, NY), pipets, 10 mL (Becton Dickinson Catalog #7551, Marietta, OH), pipets, 2 mL (Becton Dickinson Catalog #7507, Marietta, OH, pipets, 5 mL (Becton Dickinson Catalog #7543, Marietta, OH), RAW 264.7 Cells (American Type Culture Collection Catalog #TIB-71, Manassas, VA), test compounds (liquid CO<sub>2</sub> hops extract from Hopunion, Yakima, WA),  
15 (curcumin from Sigma (St. Louis, MO) (Product C 1386), 65-70% Curcuma longa powder), tissue culture plates, 96-well (Becton Dickinson Catalog #3075, Franklin Lanes, NJ), Ultra-pure water (Resistance =18 megaOhm-cm deionized water).

*General Procedure* - RAW 264.7 cells, obtained from ATCC, were grown in DMEM medium and maintained in log phase growth. The DMEM growth medium was made as follows: 50 mL of heat inactivated  
20 FBS and 5 mL of penicillin/streptomycin were added to a 500 mL bottle of DMEM and stored at 4°C. This was warmed to 37 °C in a water bath before use and for best results should be used within three months

On day one of the experiment, the log phase 264.7 cells were plated at 8 x 10<sup>4</sup> cells per well in 0.2 mL growth medium per well in a 96-well tissue culture plate. After 6 to 8 hours post plating, 100 µL of growth medium from each well was removed and replaced with 100 µL fresh medium. A 1.0 mg/mL solution of LPS,  
25 which was used to induce the expression of COX-2 in the RAW 264.7 cells, was prepared by dissolving 1.0 mg of LPS in 1 mL DMSO. It was mixed until dissolved and stored at 4 °C. Immediately before use, it was thawed at room temperature or in a 37°C water bath.

On day two of the experiment, the test materials were prepared as 1000X stock in DMSO. For example, if the final concentration of the test material was to be 10 µg/mL, a 10 mg/mL stock was prepared  
30 by dissolving 10 mg of the test material in 1 mL of DMSO. Fresh test materials were prepared on day 2 of the experiment. In 1.7 mL microfuge tubes, 1 mL DMEM without FBS was added to obtain test concentrations of 0.05, 0.10, 0.5, and 1.0 µg/mL. 2 µL of the 1000x DMSO stock of the test material was added to the 1 mL of medium without FBS. The tube contained the final concentration of the test material was concentrated 2-fold. The tube was placed in incubator for 10 minutes to equilibrate.

One-hundred mL of medium was removed from each well of the cell plates prepared on day one. One-hundred mL of equilibrated 2X final concentration the test compounds were added to cells and incubated for 90 minutes. LPS in DMEM without FBS was prepared by adding 44 µL of the 1 mg/mL DMSO stock to 10 mL of medium. For each well of cells to be stimulated, 20 µL of LPS (final concentration of LPS is 0.4 µg/mL) was added. The LPS stimulation was continued for 24 hours, after which the supernatant medium from each well was transferred to a clean microfuge tube for determination of the PGE2 content in the medium.

Determination of COX-1 Enzyme Inhibition by Curcuminoids and Hops Extract

10 The ability of a test material to inhibit COX-1 synthesis of PGE2 was determined essentially as described by Noreen, Y., et al. (J. Nat. Prod. 61, 2-7, 1998).

Equipment - balancer (2400 g, Acculab VI-2400, VWR Catalog #11237-300, Rochester, NY), balancer, analytical, Ohaus Explorer (Ohaus Model #EO1140, Switzerland), biosafety cabinet (Forma Model #F1214, Marietta, Ohio), Freezer, -30°C (Forma Model #F3797), Freezer, -80°C Ultralow (Forma Model #F8516, Marietta, OH), heated stirring plate (VWR Catalog #33918-262, Rochester, NY), ice maker (Scotsman Model #AFE400A-1A, Fairfax, SC), multichannel pipettor, 12-Channel (VWR Catalog #53501-662, Rochester, NY), Multichannel Pipettor, 8-Channel (VWR Catalog #53501-660, Rochester, NY), orbital shaker platform (Scienceware #F37041-0000, Pequannock, NJ), pH meter (VWR Catalog #33221-010, Rochester, NY), pipet aid (VWR Catalog #53498-103, Rochester, NY), pipettor, 0.5 to 10 µL (VWR Catalog #4000-200, Rochester, NY), pipettor, 100 to 1000 µL (VWR Catalog #4000-208, Rochester, NY), pipettor, 2 to 20 µL (VWR Catalog #4000-202, Rochester, NY), pipettor, 20 to 200 µL (VWR Catalog #4000-204, Rochester, NY), PURELAB Plus Water Polishing System (U.S. Filter, Lowell, MA), refrigerator, 4°C (Forma Model #F3775, Marietta, Ohio), vacuum chamber (Sigma Catalog #Z35, 407-4, St. Louis, MO), vortex mixer (VWR Catalog #33994-306, Rochester, NY)

25 Supplies and Reagents - 96-Well, round-bottom plate (Nalge Nunc #267245, Rochester, NY), arachidonic acid (Sigma Catalog #A-3925, St. Louis, MO), centrifuge tubes, 15 mL, conical, sterile (VWR Catalog #20171-008, Rochester, NY), COX-1 enzyme (ovine) 40,000 units/mg (Cayman Chemical Catalog #60100, Ann Arbor, MI), dimethylsulfoxide (DMSO) (VWR Catalog #5507, Rochester, NY), ethanol 100% (VWR Catalog #MK701908, Rochester, NY), epinephrine (Sigma Catalog #E-4250, St. Louis, MO),  
30 glutathione (reduced) (Sigma Catalog # G-6529, St. Louis, MO), graduated cylinder, 1000 mL (VWR Catalog #24711-364, Rochester, NY), hematin (porcine) (Sigma catalog # H-3281, St. Louis, MO), hydrochloric acid (HCl) (VWR Catalog #VW3110-3, Rochester, NY), KimWipes (Kimberly Clark Catalog #34256, Roswell, GA), microfuge tubes, 1.7 mL (VWR Catalog #20172-698, Rochester, NY), NaOH (Sigma Catalog #S-5881, St. Louis, MO), pipet tips for 0.5 to 10 µL pipettor (VWR Catalog #53509-138, Rochester, NY), pipet tips for 100-35 1000 µL pipettor (VWR Catalog #53512-294, Rochester, NY), pipet tips for 2-20 µL and 20-200 µL pipettors

(VWR Catalog #53512-260, Rochester, NY), prostaglandin E2 (Sigma Catalog # P-5640, St. Louis, MO), prostaglandin F2alpha (Sigma Catalog # P-0424, St. Louis, MO), stir bar, magnetic (VWR Catalog #58948-193, Rochester, NY), storage bottle, 1000 mL (Corning Catalog #1395-1L, Corning, NY), storage bottle, 100 mL (Corning Catalog #1395-100, Corning, NY), CO<sub>2</sub> extract of hops (Hopunion, Yakima, WA), curcumin 5 (Sigma, St. Louis, MO, (Product C 1386), 65-70% *Curcuma longa* powder), Tris-HCl (Sigma Catalog #T-5941, St. Louis, MO), ultra-pure water (Resistance =18 megaOhm-cm deionized water).

General Procedure - Oxygen-free 1.0M Tris-HCl buffer (pH 8.0) was prepared as follows. In a 1000 mL beaker, 12.11g Trizma HCl was dissolved into 900 mL ultra-pure water. The beaker was placed on a stir plate with a stir bar. NaOH was added until the pH reached 8.0. The volume was adjusted to a final volume of 10 1000mL and stored in a 1000 mL storage bottle.

The Tris-HCl buffer was placed into a vacuum chamber with the top loosened and the air pump was turned on until the buffer stopped bubbling. The vacuum chamber was then turned off and the storage bottle was tightly covered. This step was repeated each time when oxygen-free Tris-HCl buffer was used.

One mL cofactor solution was prepared by adding 1.3 mg (-) epinephrine, 0.3 mg reduced 15 glutathione and 1.3 mg hematin to 1 mL oxygen free Tris-HCl buffer. The solutions of the test material were prepared as needed. i.e. 10 mg of aspirin was weighed and dissolved into 1 mL DMSO.

Enzymes, i.e. prostaglandin E2 or prostaglandin F2alpha, were dissolved in oxygen free Tris-HCl buffer as follows, i.e. on ice, 6.5 µL of enzyme at 40,000 units/mL was taken and added to 643.5 µL of oxygen free Tris-HCl buffer. This enzyme solution is enough for 60 reactions. The COX-1 enzyme solution 20 was prepared as follows: In a 15 mL centrifuge tube, 10 µL COX-1 enzyme at 40,000 units/mL was added to oxygen free Tris-HCl with 50 µL of the cofactor solution per reaction. The mixture was incubated on ice for 5 minutes. For 60 reactions, 650 µL enzyme were added in oxygen free Tris-HCl buffer with 3.25 mL cofactor solution.

Sixty microliters of the enzyme solution were combined with 20 µL of the test solution in each well of 25 a 96 well plate. Final concentrations of the test solutions were 100, 50, 25, 12.5, 6.25 and 3.12 µg/mL. The plates were preincubated on ice for 10 minutes. Twenty µL arachidonic acid (30µM) was added and incubated for 15 minutes at 37°C.

Two M HCl was prepared by diluting 12.1 N HCl in a 100 mL storage bottle. 83.5 mL ultra-pure water was added and then 16.5 mL 12.1 N HCl was added. It was stored in a 100 mL storage bottle and 30 placed in the Biosafy cabinet. The reaction was terminated by adding 10 µL 2 M HCl. The final solution was used as the supernatant for the PGE<sub>2</sub> assay.

#### Determination of PGE2 Concentration in Medium

The procedure followed was that essentially described by Hamberg, M. and Samuelsson, B. (*J. Biol. Chem.* 1971. 246, 6713-6721); however a commercial, nonradioactive procedure was employed.

*Equipment* - freezer, -30°C (Forma Model #F3797), heated stirring plate (VWR Catalog #33918-262, Rochester, NY), multichannel pipettor, 12-Channel (VWR Catalog #53501-662, Rochester, NY), orbital shaker platform (Scienceware #F37041-0000, Pequannock, NJ), Pipet Aid (VWR Catalog #53498-103, Rochester, NY), pipettor, 0.5 to 10 µL (VWR Catalog #4000-200, Rochester, NY), pipettor, 100 to 1000 µL (VWR Catalog #4000-208, Rochester, NY), pipettor, 2 to 20 µL (VWR Catalog #4000-202, Rochester, NY), pipettor, 20 to 200 µL (VWR Catalog #4000-204, Rochester, NY), plate reader (Bio-tek Instruments Model #Elx800, Winooski, VT), PURELAB Plus Water Polishing System (U.S. Filter, Lowell, MA), refrigerator, 4°C (Forma Model #F3775, Marietta, Ohio).

*Chemicals, Reagents and Buffers* - Prostaglandin E<sub>2</sub> EIA Kit-Monoclonal 480-well (Cayman Chemical Catalog # 514010, Ann Arbor, MI), centrifuge tube, 50 mL, conical, sterile (VWR Catalog #20171-178, Rochester, NY), Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech Catalog #10-013-CV, Herndon, VA), graduated cylinder, 100 mL (VWR Catalog #24711-310, Rochester, NY), KimWipes (Kimberly Clark Catalog #34256, Roswell, GA), microfuge tubes, 1.7 mL (VWR Catalog #20172-698, Rochester, NY), penicillin/streptomycin (Mediatech Catalog #30-001-Cl, Herndon, VA), pipet tips for 0.5 to 10 µL pipettor (VWR Catalog #53509-138, Rochester, NY), pipet tips for 100-1000 µL pipettor (VWR Catalog #53512-294, Rochester, NY), pipet tips for 2-20 µL and 20-200 µL pipettors (VWR Catalog #53512-260, Rochester, NY), pipets, 25 mL (Becton Dickinson Catalog #7551, Marietta, OH), storage bottle, 100 mL (Corning Catalog #1395-100, Corning, NY), storage bottle, 1000 mL (Corning Catalog #1395-1L, Corning, NY), ultra-pure water (Resistance =18 megaOhm-cm deionized water).

General Procedure - EIA Buffer was prepared by diluting the contents of the EIA Buffer Concentrate (vial #4) with 90ml of Ultra-pure water. Vial #4 was rinsed several times to ensure all crystals had been removed and was then placed into a 100 mL storage bottle and stored at 4°C.

The Wash Buffer was prepared by diluting Wash Buffer Concentrate (vial #5) 1:400 with Ultra-pure water. 0.5 ml/liter of Tween 20 (vial #5a) was then added (using a syringe for accurate measurement). To prepare one liter of Wash Buffer add 2.5ml Wash Buffer Concentrate, 0.5ml Tween-20, and 997ml Ultra-pure water. The solution was stored in a 1 liter storage bottle at 4°C.

The Prostaglandin E<sub>2</sub> standard was reconstituted as follows. A 200µL pipet tip was equilibrated by repeatedly filling and expelling the tip several times in ethanol. The tip was used to transfer 100 µL of the PGE<sub>2</sub> Standard (vial #3) into a 1.7 mL microfuge tube. 900µl Ultra-pure water was added to the tube and stored at 4°C, which was stable for ~6 weeks. The Prostaglandin E<sub>2</sub> acetylcholinesterase tracer was reconstituted as follows. 100 µL PGE<sub>2</sub> tracer (vial #2) was mixed with 30 mL of the EIA Buffer in a 50 mL centrifuge tube and stored at 4°C.

The Prostaglandin E<sub>2</sub> monoclonal antibody was reconstituted as follows. 100µL PGE<sub>2</sub> Antibody (vial #1) was mixed with 30 mL of the EIA buffer in a 50 mL centrifuge tube and stored at 4°C.

DMEM with penicillin/streptomycin was prepared by adding 5 mL penicillin/streptomycin into 500 mL DMEM and stored at 4°C

The plates were set up as follows: Each plate contained a minimum of two blanks (B), two non-specific binding wells (NSB), two maximum binding wells ( $B_0$ ), and an eight point standard curve run in 5 duplicate (S1-S8). Each sample was assayed at a minimum of two dilutions and each dilution was run in duplicate.

The standard was prepared as follows: Eight 1.7 mL microuge tubes were labeled as tubes 1-8. 900  $\mu$ L DMEM into was added to tube 1 and 500  $\mu$ L DMEM to tubes 2-8. 100  $\mu$ L of the PGE<sub>2</sub> standard was added to tube 1 and mixed. Five-hundred mL of solution was taken from tube 1 and put into tube 2, and this process 10 was repeated through tube 8.

Fifty mL EIA Buffer and 50 $\mu$ L DMEM were added into the NSB wells. Fifty  $\mu$ L DMEM was added to the  $B_0$  wells. Fifty mL of solution was taken from tube #8 and added to both the lowest standard wells (S8). Fifty mL was taken from tube #7 and added to each of the next two wells. This was continued through to tube 15 #1. (the same pipet tip was used for all 8 of the standards making sure to equilibrate the tip in each new standard by pipeting up and down in that standard. Using a P200, 50 $\mu$ L of each sample at each dilution was added to the sample wells.

Using a12 channel pipetor, 50 $\mu$ L of the Prostaglandin E<sub>2</sub> acetylcholinesterase tracer was added to each well except the Total Activity (TA) and the Blank (B) wells. Using the 12 channel pipetor, 50 $\mu$ L of the Prostaglandin E<sub>2</sub> monoclonal antibody was added to each well except the Total Activity (TA), the (NSB), and 20 the Blank (B) wells. The plate was covered with plastic film (item #7) and incubated for 18 hours at 4°C.

The plates were developed as follows: one 100  $\mu$ L vial of Ellman's Reagent (vial #8) was reconstituted with 50 ml of Ultra-pure water in a 50 mL centrifuge tube. It was protected from light and used the same day. The wells were washed and rinsed five times with Wash Buffer using a 12 channel pipettor. Two-hundred mL of Ellman's Reagent was added to each well using a 12 channel pipettor and 5 $\mu$ L of Tracer 25 to the total activity(TA) wells was then added to each well using a P10 pipette. The plate was covered with a plastic film and placed on orbital shaker in the dark for 60-90 minutes.

The plate was read in the Bio-tek plate reader at a single wavelength between 405 and 420 nm. Before reading each plate, the bottom was wiped with a Kim wipe. The plate should be read when the absorbance of the wells is in the range of 0.3-0.8 A.U. If the absorbance of the wells exceeded 1.5, they were 30 washed and fresh Ellmans' Reagent was added and then redeveloped.

#### Calculation of synergy and combination index

Synergy between the curcuminoids and andrographolide was assessed using CalcuSyn (BIOSOFT, biosoft.com). This statistical package performs multiple drug dose-effect calculations using the Median Effect

methods described by T-C Chou and P. Talaly (Trends Pharmacol. Sci. 4:450-454), hereby incorporated by reference.

Briefly, it correlates the "Dose" and the "Effect" in the simplest possible form:  $fa/fu = (C/C_m)^m$ , where C is the concentration or dose of the compound and  $C_m$  is the median-effective dose signifying the potency. 5  $C_m$  is determined from the x-intercept of the median-effect plot. The fraction affected by the concentration of the test material is fa and the fraction unaffected by the concentration is fu ( $fu = 1 - fa$ ). The exponent m is the parameter signifying the sigmoidicity or shape of the dose-effect curve. It is estimated by the slope of the median-effect plot.

The median-effect plot is a plot of  $x = \log(C)$  vs  $y = \log(fa/fu)$  and is based on the logarithmic form of 10 Chou's median-effect equation. The goodness of fit for the data to the median-effect equation is represented by the linear correlation coefficient r of the median-effect plot. Usually, the experimental data from enzyme or receptor systems have an  $r > 0.96$ , from tissue culture an  $r > 0.90$  and from animal systems an  $r > 0.85$ .

Synergy of test components is quantified using the combination index (CI) parameter. The CI of Chou-Talaly is based on the multiple drug-effect and is derived from enzyme kinetic models (Chou, T.-C. and 15 Talalay, P. (1977) A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. J. Biol. Chem. 252:6438-6442). The equation determines only the additive effect rather than synergism or antagonism. However, we define synergism as a more than expected additive effect, and antagonism as a less than expected additive effect as proposed by Cho and Talalay in 1983 (Trends Pharmacol. Sci. (1983) 4:450-454). Using the designation of  $CI = 1$  as the additive effect, we obtain for 20 mutually exclusive compounds that have the same mode of action or for mutually non-exclusive drugs that have totally independent modes of action the following relationships:  $CI < 1$ ,  $= 1$ , and  $> 1$  indicating synergism, additivity and antagonism, respectively.

Expected median inhibitory concentrations of the two-component combinations were estimated using 25 the relationship:

$$[1/\text{Expected } IC_{50}] = [A/IC_{50}A] + [B/IC_{50}B]$$

where A = mole fraction of component A in the combination and B = the mole fraction of component B in the combination.

TABLE 4 illustrates the observed and expected median inhibitory concentrations for curcumin and hops extract for PGE2 production by COX-2 in the RAW 264.7 cell assay. While the expected  $IC_{50}$  for the 30 10:1 combination of curcumin and hops extract was 1.6  $\mu\text{g/mL}$ , the observed value was 0.77  $\mu\text{g/mL}$  or 2-fold greater. This level of difference was unexpected and constitutes a novel finding for the combined COX-2 inhibitory activity of the 1:10 combination of curcumin and hops extract.

TABLE 4. Observed and Expected Median Inhibitory Concentrations for a (10:1) Formulation of Curcumin and Hops Extract

Composition	Ratio	IC <sub>50</sub> (µg/ml)
Hops Extract		0.216
Curcumin		4.5
<u>Combined</u>		
Hops Extract Contribution	1	0.071
Curcumin Contribution	10	0.715
Observed		0.786
Calculated		1.605

5 Statistical analysis of inhibition of COX-2 production of PGE2 in the RAW 264.7 cell model for the 1:10 combination of curcumin and hops extract is presented in TABLE 5. The CI for this combination was 0.490, 0.472 and 0.454, respectively, for the IC<sub>50</sub>, IC<sub>75</sub> and IC<sub>90</sub>. These CI values indicate strong synergy between curcumin and hops extract over the complete dose-response curve.

10 TABLE 5. Combination Index for a 1:10 Formulation of Curcumin and Hops Extract

IC <sub>50</sub>	Combination Index		Mean CI
	IC <sub>75</sub>	IC <sub>90</sub>	
0.490	0.472	0.454	0.472

The medium inhibitory concentration of COX-2 by curcumin alone in the RAW 264.7 cell model was 4.01 µg/mL (TABLE 6). Inhibition of COX-1 enzyme activity by curcumin was somewhat higher with an IC<sub>50</sub> of 10.0 µg/mL. Hops extract exhibited an IC<sub>50</sub> of PGE2 inhibition by COX-2 of 0.21 µg/mL and an IC<sub>50</sub> for COX-1 enzyme inhibition estimated at 6.25 µg/mL; the COX-2 specificity of curcumin alone was 2.5 and for hops extract, it was 29.5. Eleven formulations of curcumin and hops extract exhibited COX-2 specificity ranging from 48.6 to 11.2, with a median COX-2 specificity of 17.4. All of the combinations of curcumin and hops extract unexpectedly demonstrated COX-2 specificity greater than the nominal 5.0 suggested as the minimum for pharmaceutical products designed to limit PGE2 production specifically through inhibition of COX-2. This finding indicates that combinations of curcumin and a hops extract could function as potent anti-inflammatory formulations without the GI side effects seen with COX-1 inhibition.

**TABLE 6. COX-2 Specificity for Curcumin, Hops Extract and Eleven Formulations of Curcumin and Hops Extract**

Hops Extract: Curcumin [x:y]	Hops Extract [%]	Curcumin [%]	COX-1 IC <sub>50</sub> [µg/ml]	COX-2 IC <sub>50</sub> [µg/ml]	COX-1/ COX-2
	100	0	6.25	0.212	29.5
[10:1]	91	9	6.471	0.186	34.8
[8:1]	89	11	6.522	0.426	15.3
[6:1]	86	14	6.604	0.590	11.2
[4:1]	80	20	6.757	0.389	17.4
[2:1]	67	33	7.143	0.147	48.6
[1:1]	50	50	7.692	0.452	17.0
[1:2]	33	67	8.333	0.332	25.1
[1:4]	20	80	8.929	0.377	23.7
[1:6]	14	86	9.211	0.449	20.5
[1:8]	11	89	9.375	0.563	16.7
[1:10]	9	91	9.483	0.786	12.1
	0	100	10.0	4.01	2.5

5

### EXAMPLE 2

## Normalization of Joint Functioning Following Trauma

A representative composition of the present invention as a dietary supplement would be in an oral formulation, i.e. tablets, that would supply one of the following combinations: (a) 15 mg curcuminoid/kg per day and 6.0 mg humulone/kg per day; (b) 15 mg curcuminoid/kg per day and 6.0 mg upulons/kg per day; (c) 15 mg curcuminoid/kg per day and 6.0 mg dihydroisohumulones/kg per day. Normalization of joint movement following physical trauma due to exercise or repetitive movement stress would be expected to occur following two to ten doses. This result would be expected in all animals.

15

### EXAMPLE 3

## Clinical Effectiveness of Lotion Formulations in the Treatment of Acne Rosacea

A lotion designed to contain one of the following: (a) 0.1% wt curcuminoids and 0.5% humulone; or (b) 0.1% wt curcuminoids and 0.5% lumulone is applied to affected areas of patients who have exhibited acne rosacea as diagnosed by their health practitioner and confirmed by an independent board-certified

dermatologist. Self-evaluation tests are administered one week prior to the study to quantify the surface area affected and redness. In addition, similar variables are scored by the professional clinical staff not aware of the patients treatment status. These evaluations are repeated on Days 0, 7, 14 and 21.

Patients are randomly assigned to the test formulation or placebo at the start of the study. The test formulation and placebo are applied to the affected area one or two times per day. Treatment for health conditions such as diabetes, hypertension, etc. is allowed during the study. Scores are statistically compared between the test formulation and the placebo for each of the four observational periods. Patients treated with the composition of the present invention in a lotion formulation are considered improved if the patients' scores improve by greater than 20% from the pre-test scores within each category evaluated. The percentage of persons exhibiting improvement is compared between the combination formulations and the placebo control. The difference between the two groups is considered statistically significant if the probability of rejecting the null hypothesis when true is less than five percent.

#### EXAMPLE 4

##### 15 Clinical Effectiveness of Lotion Formulation in the Treatment of Psoriasis

This example is performed in the same manner as described in Example 3, except that the composition is applied to affected areas of patients who have exhibited psoriasis as diagnosed by their own practitioner and confirmed by an independent board-certified dermatologist. Self-evaluation tests are administered one week prior to the study to quantify the surface area affected and skin condition. In addition, similar variables are scored by the professional clinical staff not aware of the patients treatment status. These evaluations are repeated on Days 0, 7, 30 and 60.

Patients are randomly assigned to the test formulation or placebo at the start of the study. The test formulation and placebo are applied to the affected area one or two times per day. Treatment for health conditions such as diabetes, hypertension, etc. is allowed during the study. Scores are statistically compared between the test formulation and the placebo for each of the four observational periods. Patients treated with the composition of the present invention as the test lotion formulation are considered improved if the patients' scores improve by greater than 20% from the pre-test scores within each category evaluated. The percentage of persons exhibiting improvement is compared between the test formulation and the placebo control. The difference between the two groups is considered statistically significant if the probability of rejecting the null hypothesis when true is less than five percent.

EXAMPLE 5Clinical Effectiveness of a Formulation in the Treatment of Alzheimer's Disease

An oral formulation as described in Example 2 is administered to patients who have manifested an early stage of Alzheimer's Disease (AD), as diagnosed by their practitioner and confirmed by an independent board-certified neurologist. Two weeks before the clinical trial, the patients undergo appropriate psychoneurological tests such as the Mini Mental Status Exam (MMSE), the Alzheimer Disease Assessment Scale (ADAS), the Boston Naming Test (BNT), and the Token Test (TT). Neuropsychological tests are repeated on Day 0, 6 weeks and 3 months of the clinical trial. The tests are performed by neuropsychologists who are not aware of the patient's treatment regimen.

Patients are randomly assigned to the test formulation or placebo at the start of the study. The test formulation and placebo are taken orally one or two times per day. Treatment for conditions such as diabetes, hypertension, etc. is allowed during the study. Scores are statistically compared between the test formulation and the placebo for each of the three observational periods. Without treatment, the natural course of AD is significant deterioration in the test scores during the course of the clinical trial. Patients treated with the composition of the present invention as the test formulation are considered improved if the patients' scores remain the same or improve during the course of the clinical trial.

EXAMPLE 6Oral Formulation in the Treatment and Prevention of Colon Cancer

An oral formulation as described in Example 2 is administered to patients who have manifested an early stage of colon cancer as diagnosed by their own practitioner and confirmed by a independent board-certified oncologist.

Patients are randomly assigned to the test formulation or a placebo at the start of the study. The test formulation and placebo are taken orally one or two times per day. Treatment for conditions such as diabetes, hypertension, etc. is allowed during the study. Endoscopic evaluations are made at one, two, six and twelve months. Evidence of reappearance of the tumor during any one of the four follow-up clinical visits is considered a treatment failure. The percentage of treatment failures is compared between the test formulation and the placebo control. Under the experimental conditions described, the test material is expected to decrease the tumor incidence with respect to the control group. The difference between the two groups is considered statistically significant if the probability of rejecting the null hypothesis when true is less than five percent.

EXAMPLE 7Oral Formulation for the Treatment of Irritable Bowel Syndrome

An oral formulation as described in Example 2 is administered to patients who have manifested 5 irritable bowel syndrome as diagnosed by their practitioner. Normal bowel functioning is restored within 24 hours.

EXAMPLE 8Normalization of Joint Functioning in Osteoarthritis

10 Using compositions described in Example 2 normalization of joint stiffness due to osteoarthritis occurs following five to twenty doses, in the presence or absence of glucosamine or chondroitin sulfate. In addition, the composition does not interfere with the normal joint rebuilding effects of these two proteoglycan constituents, unlike traditional non-steroidal anti-inflammatory agents.

15 In summary, a certain embodiment is a composition for inhibition of inducible COX-2 activity and having minimal effect on COX-1 activity, said composition comprising, as a first component an effective amount of a curcuminoid species and an effective amount of a second component selected from the group consisting of an alpha-acid species and a beta-acid species or derivatives thereof. The curcuminoid species is preferably curcumin, demethoxycurcumin, or bisdemethoxycurcumin. The alpha-acid species is preferably 20 humulone, cohumulone, isohumulone, isoprehumulone, hulupone, adhumulone, xanthohumol A, or xanthohumol B. The beta-acid species is preferably lupulone, colupulone, adlupulone, tetrahydroisohumulone, hexahydrocolupulone or dihydro-isohumulone. The first or the second components of the present composition may be of pharmaceutical grade or derived from plant(s) or plant extract(s). The first or second components may also be conjugated with a compounds such as mono- or di- saccharides, amino acids, sulfates, succinates, acetates or glutathione. The compositions of the preferred embodiments can be formulated in a pharmaceutically acceptable carrier and contain additives, such as antioxidants, vitamins, minerals, proteins, fats, carbohydrates, glucosamine, chondroitin sulfate or aminosugars.

Other embodiments include methods of dietary supplementation of the compositions of the preferred embodiments to reduce the symptoms in animals suffering from symptoms of inflammation. The composition 30 is formulated in a dosage form such that said administration provides from about 0.001 to about 30.0 mg body weight per day of each curcuminoid species, and from about 0.5 to about 20.0 mg/kg bodyweight per day of alpha-acid species or beta-acid species. The composition is administered in an amount sufficient to maintain a serum concentration of about 0.1 to about 50  $\mu$ M of each curcuminoid species, and from about 0.001 to about 50  $\mu$ M of each alpha-acid species or beta-acid species. The animal may be humans, non-human

primates, dogs, cats, birds, reptiles, amphibians, horses or ruminants. The administration may be an oral, parenteral, topical, transdermal or transmucosal delivery system.

Thus, among the various formulations taught there has been disclosed a formulation comprising curcuminoids, as the first component, and a second compound selected from the group consisting of alpha-acids and beta-acids. These combinations can provide for a synergistic anti-inflammatory effect in response to physical or chemical injury or abnormal immune stimulation due to a biological agent or unknown etiology. It will be readily apparent to those skilled in the art that various changes and modifications of an obvious nature may be made without departing from the spirit of the invention, and all such changes and modifications are considered to fall within the scope of the invention as defined by the appended claims.

10

WHAT IS CLAIMED IS:

1. A composition comprising an effective amount of a first component comprising a curcuminoid species and a second component comprising a member selected from the group consisting of an alpha acid, a beta acid, and derivatives thereof.
- 5 2. The composition of Claim 1, wherein the alpha acid is selected from the group consisting of humulone, cohumulone, isohumulone, isoprehumulone, hulupone, adhumulone, xanthohumulone A, and xanthohumulone B.
3. The composition of Claim 2, wherein the alpha acid is humulone.
4. The composition of Claim 1, wherein the beta acid is selected from the group consisting of 10 lupulone, colupulone, adlupulone, tetrahydroisohumulone, hexadydrocolupulone, and dihydroisohumulone.
5. The composition of Claim 4, wherein the beta acid is lupulone.
6. The composition of Claim 1, wherein the alpha acid or beta acid is conjugated to a compound selected from the group consisting of mono- or di- saccharides, amino acids, fatty acids, sulfates, succinate, acetate, and glutathione.
- 15 7. The composition of Claim 1, wherein the second component is extracted from hops.
8. The composition of Claim 7, wherein the extraction is performed by supercritical CO<sub>2</sub>.
9. The composition of Claim 1, wherein the curcuminoid is selected from the group consisting of curcumin, demethoxycurcumin, bisdemethoxycurcumin, cis-trans-curcumin, and cyclocurcumin.
10. The composition of Claim 9, wherein the curcuminoid is curcumin.
- 20 11. The composition of Claim 1, wherein the curcuminoid is conjugated to a compound selected from the group consisting of mono- or di- saccharides, amino acids, fatty acids, sulfates, succinate, acetate, and glutathione.
12. The composition of Claim 1 wherein the first component is a synthetic compound.
13. The composition of Claim 1 wherein the second component is a synthetic compound.
- 25 14. The composition of Claim 1, wherein the composition is formulated with a pharmaceutically acceptable carrier.
15. The composition of Claim 1, further comprising a member selected from the group consisting of glucosamine and chondroitin sulfate.
16. The composition of Claim 1, further comprising a member selected from the group 30 consisting of antioxidants, vitamins, minerals, proteins, fats, carbohydrates, and aminosugars.
17. The composition of Claim 1 for use in treating inflammation or inflammation-based diseases in an animal.
18. The composition of Claim 1 for use reducing the symptoms of osteoarthritis in an animal.
19. Use of the composition of Claim 1 for the preparation of a medicament for treating 35 inflammation or inflammation-based diseases in an animal.

20. The use of Claim 19, wherein the composition is formulated in a dosage form such that said administration provides about 0.001 to about 30 mg per kg body weight of the animal per day of the first component and about 0.5 to about 20 mg per kg body weight of the animal per day of the second component.

21. The use of Claim 19, wherein administration is by a means selected from the group consisting of oral, parenteral, topical, transdermal, and transmucosal delivery.  
5

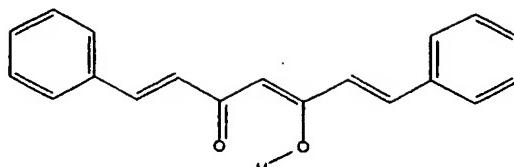
22. The use of Claim 21, wherein the topical application formula provides about 0.001 to about 1 wt% of the first component and about 0.025 to about 1 wt% of the second component.

23. The use of Claim 22, wherein the topical application formula provides about 0.01 to about 1 wt% of the first component and about 0.05 to about 1 wt% of the second component.

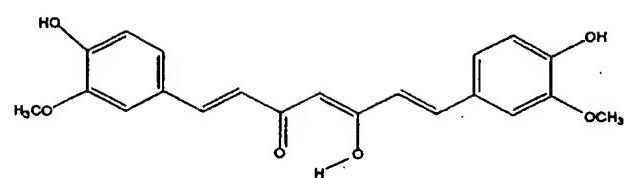
10 24. Use of the composition of Claim 1 for the preparation of a medicament for reducing the symptoms of osteoarthritis in an animal.

25. The use of Claim 24, wherein the composition further comprises a member selected from the group consisting of glucosamine and chondroitin sulfate.

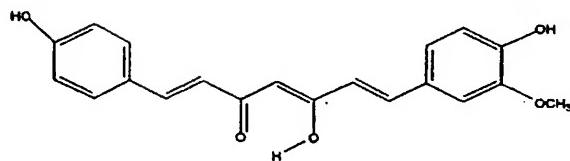
[A]



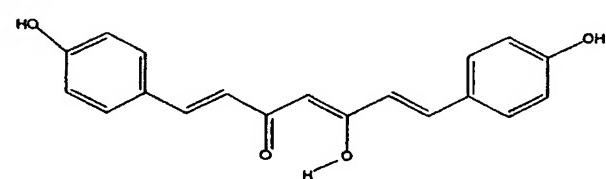
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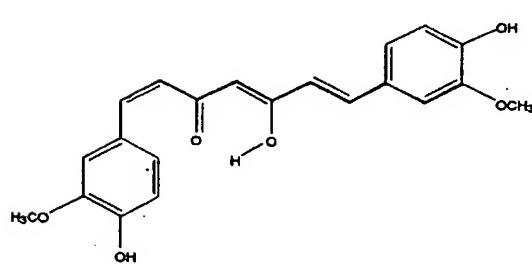
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[D]



[E]



[F]

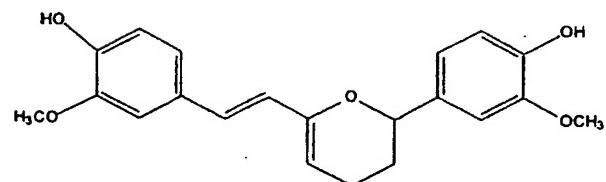
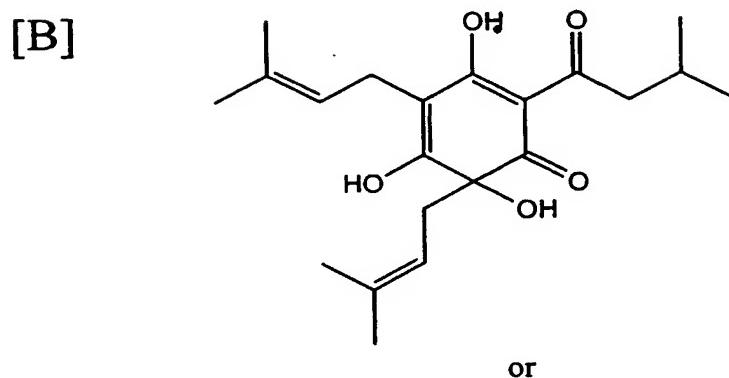
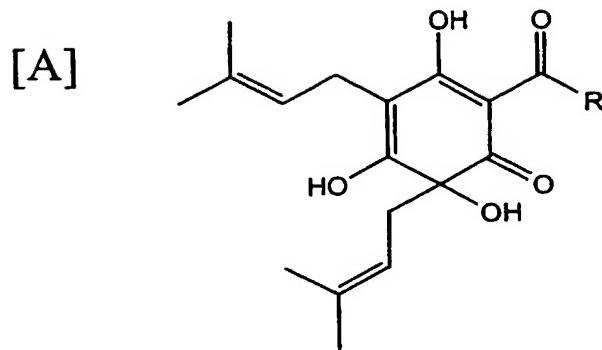


Fig. 1



or

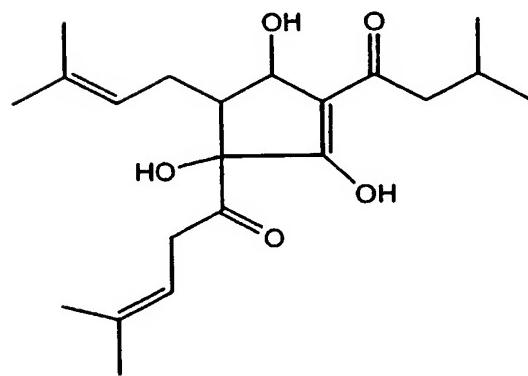
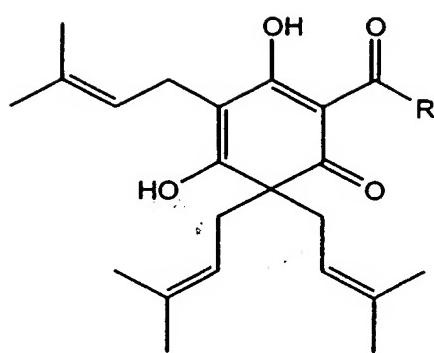


Fig. 2

3 / 3

[A]



[B]

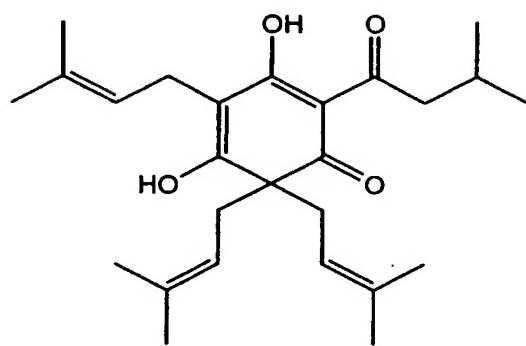


Fig. 3

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60/335,062 26 October 2001 (26.10.2001) US
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- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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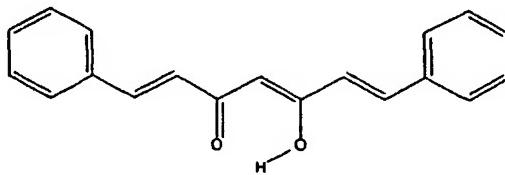
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18 September 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**WO 03/035007 A3**

(54) Title: CURCUMINOID COMPOSITIONS EXHIBITING SYNERGISTIC INHIBITION OF THE EXPRESSION AND/OR ACTIVITY OF CYCLOOXYGENASE-2



(57) Abstract: A novel formulation is provided that serves to inhibit the inflammatory response in animals. The formulation comprises, as a first component an effective amount of a curcuminoid species and an effective amount of a second component selected from the group consisting of an alpha-acid species or a beta-acid species or derivatives thereof. The composition provides synergistic anti-inflammatory effects in response to physical or chemical injury or abnormal immune stimulation due to a biological agent or unknown etiology.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/34456

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 35/78  
US CL : 424/756

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
U.S. : 424/756

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 6,391,346 B1 (NEWMARK et al) 21 May 2002 (21.05.2002), abstract, Table 1.	1-25
Y	US 5,494,668 (PATWARDHAN) 27 February 1996 (27.02.1996), column 5, lines 4-10.	1-25
Y	US 6,143,303 (JANAKIRAM et al) 07 November 2000 (07.11.2000), column 1, lines 6-14.	1-25
Y,P	US 6,346,519 B1 (PETRUS) 12 February 2002 (12.02.2002), example 1.	1-25

Further documents are listed in the continuation of Box C.



See patent family annex.

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later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

30 May 2003 (30.05.2003)

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PCT/US02/34456

INTERNATIONAL SEARCH REPORT

**Continuation of B. FIELDS SEARCHED Item 3:**

Searched West: (curcuminoid or curcumin or turmeric or curcuma or curcum\$) same (humulone or cohumulone or isohumulone or isoprehumulone or hulupone or adhumulone or xanthohumulone or xanthohumulone) same (hops o lupulone or colupulone or adlupulone or tetrahydroisohumulone or hexahydrocolupulone or dihydroisohumulone)

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